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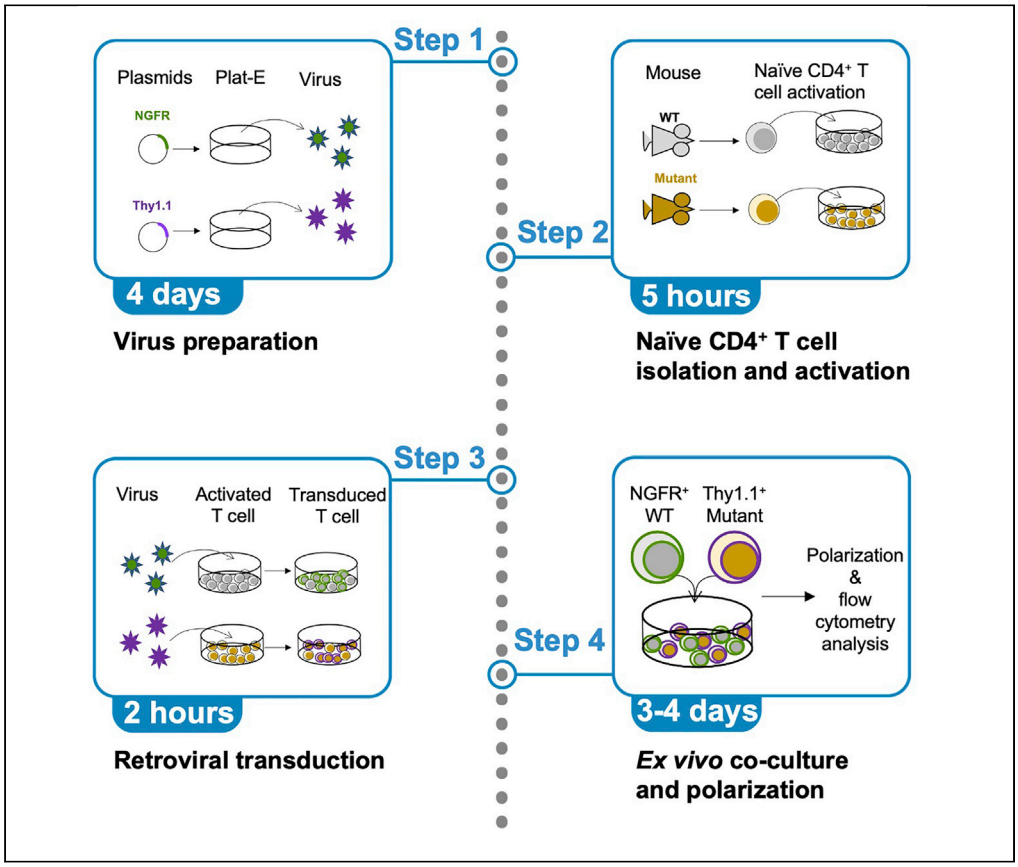
Protocol to assess cell-intrinsic regulatory mechanisms using an *ex vivo* murine T cell polarization and co-culture system



This protocol describes an *ex vivo* cell culture system for simultaneously generating a mixture of $CD4^+$ T helper lineages, including T helper 17 (Th17), $ROR\gamma t^+$ Treg, and conventional Treg (cTreg), in proportions representative of those found in mucosal tissues *in vivo*. When combined with a co-culture approach, this system allows a more rapid assessment of a candidate molecule's T cell-intrinsic and -extrinsic functions over the traditional bone marrow chimera and co-transfer approaches.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Approach to generate physiologically relevant heterogeneous T cell populations

Simultaneously evaluate effector and suppressor functions of multiple T cell subsets

Rapidly define cell-intrinsic and -extrinsic roles of target molecules *ex vivo*

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Protocol to assess cell-intrinsic regulatory mechanisms using an *ex vivo* murine T cell polarization and co-culture system

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SUMMARY

This protocol describes an *ex vivo* cell culture system for simultaneously generating a mixture of CD4$^+$ T helper lineages, including T helper 17 (Th17), RORγt$^+$ Treg, and conventional Treg (cTreg), in proportions representative of those found in mucosal tissues *in vivo*. When combined with a co-culture approach, this system allows a more rapid assessment of a candidate molecule's T cell-intrinsic and -extrinsic functions over the traditional bone marrow chimera and co-transfer approaches.

For complete details on the use and execution of this protocol, please refer to Ma et al. (2022).

BEFORE YOU BEGIN

For experiments involving the use of retrovirus, it is important to follow the universal safety precautions outlined in (Coffin et al., 1997), including the use of a class II biological safety cabinet, lab coat, protective sleeves, and double gloves. Decontamination can be carried out with standard detergent or 70% ethanol (Coffin et al., 1997).

The retroviral vectors described in this protocol are detailed in Figure 1. The workflow of this protocol is outlined in Figure 2. Some steps are modified from previous reports (Eremenko et al., 2021; Flaherty and Reynolds, 2015). In this section, we describe the steps for preparing the retroviral constructs, culturing the Plat-E retrovirus packaging cell line, and obtaining the appropriate mice before the start of T cell isolation and culture.

DNA plasmid preparation

Timing: 2–3 days

This section prepares retroviral constructs encoding expression cassettes for unique cell surface molecules that will be used for distinguishing T-cells from distinct sources in the co-culture assay.

1. Transformation and culturing of *E. coli*.
   a. 50 μL DH5α *E. coli* is transformed with 50 ng of either MSCV-IRES-Thy1.1 (Thy1.1) or MSCV-IRES-NGFR (NGFR) -encoding plasmids by heat shock at 42°C for 30 seconds.
   b. The transformed DH5α cells are evenly plated on a Luria-Bertani (LB) agar plate with 100 μg/mL ampicillin and incubated at 37°C overnight.

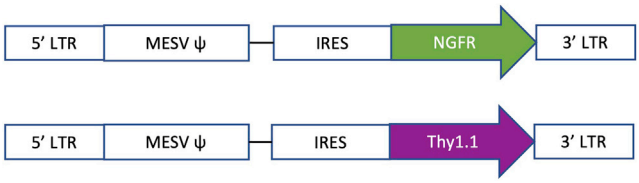

Figure 1. Schematic representation of the retroviral vectors described in this protocol
Top: MSCV-IRES-NGFR (NGFR, Addgene #27489). Bottom: MSCV-IRES-Thy1.1 (Thy1.1, Addgene #17442).

c. One transformed *E. coli* clone (single colony) is picked and grown overnight in 50 mL LB medium containing 100 μg/mL ampicillin with agitation (250 rpm) at 37°C.
d. Final spectrophotometer assessment at the 600 nm absorbance (A600) of 1–1.5 will yield ~10^9 Colony Forming Units (CFU)/mL.
e. Pellet bacteria following the manufacturer's instructions for the PurelinkTM HiPure Plasmid Filter Midiprep Kit [Life Technologies, K210005] or equivalent bacterial Midiprep kit to isolate plasmids from bacterial cultures prepared in step 1.

2. DNA concentration of the purified plasmids is determined using a spectrophotometer (e.g., Nanodrop).

Establishment of Platinum-E (Plat-E) cell culture

Timing: 1 week

The Plat-E retroviral packaging cell line was derived from the 293T cell line. Plat-E cells exhibit longer stability and produce higher yields of retroviral structure proteins than conventional NIH3T3-based cell lines. These cells already express *gag*, *pol*, and *env* genes encoding the retroviral structure proteins, allowing retroviral packaging with transfection of a single plasmid.

3. Recovery of frozen Plat-E cell stock.
   a. Obtain a Plat-E cell stock vial from −80°C or liquid nitrogen storage.

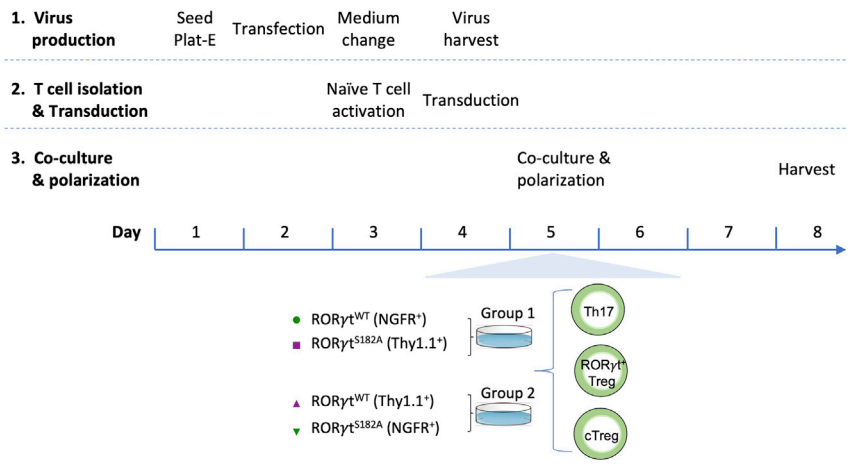

Figure 2. Schematic overview of the murine splenic T-cell retroviral transduction and co-culture protocol
Step 1: Production of Thy1.1 and NGFR-expression construct-carrying retroviruses in the Plat-E packaging cell line. Step 2: Isolation, activation, and transduction of splenic naïve $CD4^+$ T cells from $ROR\gamma t^{WT}$ and $ROR\gamma t^{S182A}$ mice. Step 3: Co-culture, polarization, and harvest of retrovirally transduced $ROR\gamma t^{WT}$ and $ROR\gamma t^{S182A}$ $CD4^+$ T cells marked by cell surface Thy1.1 or NGFR.

b. Seed cells onto a 10 cm^2 plate or a 75 cm^2 flask with 10 mL Plat-E medium pre-warmed to room temperature (RT).
c. Incubate at 37°C in a humidified 5% CO_2-containing incubator.

4. Post recovery, cells should be passaged 1–2 times when confluency reaches ~80%.
5. After the second passage, cells are now optimal for transfection and virus production.

Mouse models

The following protocol describes one recent application of this assay for evaluating the cell-intrinsic role of a post-translational modification on a master T cell transcription factor RORγt. For this experiment, naïve $CD4^+$ T cells are obtained from 8–14 weeks old gender-matched and co-housed $ROR\gamma t^{WT}$ and $ROR\gamma t^{S182A}$ littermates in C57BL/6 background. "$ROR\gamma t^{WT}$" refers to wild-type mice. "$ROR\gamma t^{S182A}$" refers to a phospho-null knock-in mouse line generated by replacing the serine codon on *Rorc* with that of alanine using CRISPR-Cas9. For more information on the mouse models used in this protocol, please refer to (Ma et al., 2022).

Institutional permissions

All animal studies followed guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego. Readers wishing to follow this protocol must first obtain approval from the official animal use ethical committee of their respective institutions to perform the animal studies described herein.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
DMEM	Thermo Fisher Scientific	Cat# 11960044
Iscove's Modified Dulbecco's Medium (IMDM)	Sigma-Aldrich	Cat# I3390
Opti-MEM™ I Reduced Serum Medium	Life Technologies	Cat# 31985062
Fetal Bovine Serum (FBS)	Peak Serum	Cat# PS-FB3
L-Glutamine-200 mM (100×)	Thermo Fisher Scientific	Cat# 250-30-081
β-mercaptoethanol	Thermo Fisher Scientific	Cat# 21985023
Gentamycin	Sigma-Aldrich	Cat# G1272
Penicillin-streptomycin	Thermo Fisher Scientific	Cat# 10378016
ACK Lysing Buffer	Gibco	Cat# A1049201
PBS (1×) pH 7.4	Thermo Fisher Scientific	Cat# 10010-049
Polybrene Infection / Transfection Reagent	Sigma-Aldrich	Cat# TR-1003-G
TransIT®-293 Transfection Reagent	Mirus	Cat# MIR2700
0.05% Trypsin-EDTA (1×), phenol red	Life Technologies	Cat# 25300054
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	Cat# P8139
Ionomycin	Sigma-Aldrich	Cat# I0634
GolgiSTOP	BD	Cat# 554724, RRID: AB_2869012
Ampicillin	Sigma-Aldrich	Cat# A0166
Luria Broth Base	Invitrogen	Cat# 12795-027
LB Agar	Invitrogen	Cat# 22700-025
0.5 M EDTA	Invitrogen	Cat# AM9260G
LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, 405 nm (Dilution 1:500)	Thermo Fisher Scientific	Cat# L34957
Recombinant Human TGF-beta 1 Protein	R&D	Cat# 240-B
Recombinant Mouse IL-6 Protein	R&D	Cat# 406-ML
Recombinant Mouse IL-1β Protein	R&D	Cat# 401-ML
Critical commercial assays		
PureLink® HiPure Plasmid Midiprep Kit	Life Technologies	Cat# K210005
Naive $CD4^+$ T Cell Isolation Kit, mouse	Miltenyi Biotec	Cat# 130-104-453
eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set	Thermo Fisher Scientific	Cat# 00-5523-00

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cell lines and bacteria strain		
Human: Plat-E Retroviral Packaging Cell Line	Cell Biolabs, Inc.	Cat# RV-101
DH5α *E. coli*	Biomiga	Cat# CC1101-01
Experimental models: Organisms/strains		
C57BL/6 RORγt^{WT} and RORγt^{S182A} mice (gender-matched 8–12-week-old littermates)	Ma et al., (2022)	N/A
Recombinant DNA		
MSCV-IRES-NGFR (NGFR)	Addgene	RRID: Addgene_27489
MSCV-IRES-Thy1.1 (Thy1.1)	Addgene	RRID: Addgene_17442
Antibodies		
Rabbit IgG Fraction to Hamster IgG (Whole Molecule) Antibody (Dilution 1:20)	MP Bio	Cat# 0855398, RRID: AB_2334935
PE anti-human CD271 (NGFR) Antibody (Clone ME20.4) (Dilution 1:400)	BioLegend	Cat# 345106, RRID: AB_2152647
Pacific Blue™ anti-rat CD90/mouse CD90.1 (Thy-1.1) Antibody (clone OX-7) (Dilution 1:400)	BioLegend	Cat# 202521, RRID: AB_1595584
APC/Cyanine7 anti-mouse IL-17A Antibody (clone TC11-18H10.1) (Dilution 1:100)	BioLegend	Cat# 506939; RRID: AB_2565780
PE/Cyanine5 anti-mouse CD4 Antibody (clone GK1.5) (Dilution 1:400)	BioLegend	Cat# 100410; RRID: AB_312695
CD3ε Monoclonal Antibody (clone 145-2C11), Functional Grade (Dilution 1:1000)	Thermo Fisher Scientific	Cat# 16-0031-86, RRID: AB_468849
CD28 Monoclonal Antibody (clone 37.51), Functional Grade (Dilution 1:4000)	Thermo Fisher Scientific	Cat# 16-0281-86, RRID: AB_468923
ROR gamma (t) Monoclonal Antibody (clone B2D), PE-eFluor 610 (Dilution 1:200)	Thermo Fisher Scientific	Cat# 61-6981-82; RRID: AB_2574650
IL-10 Monoclonal Antibody (clone JES5-16E3), Alexa Fluor 488 (Dilution 1:100)	Thermo Fisher Scientific	Cat# 53-7101-80; RRID: AB_469925
IL-10 Monoclonal Antibody (clone JES5-16E3), Alexa Fluor 700 (Dilution 1:100)	Thermo Fisher Scientific	Cat# 56-7101-80; RRID: AB_891570
FOXP3 Monoclonal Antibody (clone FJK-16s), PE-Cyanine5.5 (Dilution 1:200)	Thermo Fisher Scientific	Cat# 35-5773-82; RRID: AB_11218094
ChromPure Rat IgG (Dilution 1:50)	Jackson ImmunoResearch	Cat# 012-000-013; RRID: AB_2337136
Software and algorithms		
FlowJo™ v10.8	BD	https://www.flowjo.com/
Other		
Biological Safety Cabinet	Thermo Fisher Scientific	1384-M
Centrifuge	Eppendorf	5810R
NanoDrop Lite	Thermo Fisher Scientific	BZB8917251
ZE5 Cell Analyzer	Bio-Rad	12004278
15 mL Conical tubes	Corning	Cat# 188261
50 mL Conical tubes	Corning	Cat# 227270
24-well tissue culture plate	Fisher Scientific	Cat# 353047
6-well tissue culture plate	Eppendorf	Cat# 30720113
10 mL serological pipettes	Genesee Scientific	Cat# 12-112
5 mL serological pipettes	Genesee Scientific	Cat# 12-110
3 mL syringe	Fisher Scientific	Cat# 14823436
Millex-HV Syringe Filter Unit, 0.45 μm	Fisher Scientific	Cat# SLHV033RS
40 μm cell strainer	Falcon	Cat# 352340
Curved forceps	Denville	Cat# S728685
Delicate tip straight forceps	Denville	Cat# S728696
Micro dissecting scissors 3.5″	Roboz Surgical Store	Cat# RS-5910
Vi-Cell cell counter	Beckman Coulter	AZ41595

Alternatives: In theory, all reagents and resources listed in the 'key resources table' can be substituted with equivalent items from other suppliers; however, it should be noted that the protocol has been calibrated to the reagents listed in the above table and alternatives have not been tested on the protocol performance.

MATERIALS AND EQUIPMENT

LB medium	
Reagent	Amount
Luria Broth Base	25 g
MilliQ H2O	Up to 1 L
Total	**1 L**
Autoclave on a liquid cycle (20 min at 15 psi), and then store at RT for up to 3 months.	

LB agar plate with ampicillin	
Reagent	Amount
Luria Broth Base	12.5 g
Agar	7.5 g
MilliQ H2O	Up to 0.5 L
Total	**0.5 L**
Autoclave on a liquid cycle (20 min at 15 psi). Allow the agar solution to cool to 55°C–60C, then add 500 µL of 100 mg/mL ampicillin and swirl to mix. Pour 20 mL of LB agar per 10 cm^2 plate and allow plates to cool and solidify. Store at 4°C for up to 2 months.	

Plat-E medium		
Reagent	Final concentration	Amount
Fetal Bovine Serum	10% (v/v)	5 mL
L-Glutamine (200 mM)	2 mM	0.5 mL
DMEM	n/a	44.5 mL
Total	**n/a**	**50 mL**
Prepare in a biological safety cabinet. Store at 4°C for up to one month.		

IMDM complete medium		
Reagent	Final concentration	Amount
L-Glutamine (200 mM)	2 mM	1 mL
β-mercaptoethanol (55 mM)	55 µM	50 µL
Gentamycin (10 mg/mL)	50 µg/mL	250 µL
Penicillin-streptomycin (100×)	1 mg/mL Streptomycin, 100 U/mL Penicillin	500 µL
Fetal Bovine Serum	10% (v/v)	5 mL
IMDM	n/a	43.2 mL
Total	**n/a**	**50 mL**
Prepare in a biological safety cabinet. Store at 4°C for up to two weeks.		

⚠ CRITICAL: β-mercaptoethanol is toxic. It may cause severe skin, eye, and respiratory tract irritation if absorbed or inhaled. Always wear personal protective equipment (PPE) when handling and use only inside a well-ventilated safety cabinet or fume hood.

T-cell polarization medium		
Reagent (stock concentration)	Final concentration	Amount
anti-CD3ϵ (1000 μg/mL)	0.25 μg/mL	2.5 μL
anti-CD28 (1000 μg/mL)	1 μg/mL	1 μL
IL-6 (10 μg/mL)	20 ng/mL	2 μL
TGFβ (2 μg/mL)	5 ng/mL	2.5 μL
IL-1β (10 μg/mL)	20 ng/mL	2 μL
IMDM	n/a	990 μL
Total	**n/a**	**1 mL**
Prepare in a biological safety cabinet. Use immediately.		

MACS buffer		
Reagent	Final concentration	Amount
Fetal Bovine Serum	2% (v/v)	1 mL
EDTA (500 mM)	2 mM	0.2 mL
PBS (1×)	n/a	48.8 mL
Total	**n/a**	**50 mL**
Prepare in a biological safety cabinet. Store at 4°C for up to one month.		

Antibodies cocktail n°1 – Surface markers post-restimulation – 1× concentration		
Reagent (stock concentration)	Amount	Working dilution (in antibodies cocktail)
LIVE/DEAD Fixable Aqua (500×)	0.2 μL	1:500
CD4 PE-Cy5 (0.2 mg/mL)	0.25 μL	1:400
Thy1.1 Pacific Blue (0.5 mg/mL)	0.25 μL	1:400
NFGR PE (0.05 mg/mL)	0.25 μL	1:400
PBS (1×)	Up to 100 μL	
Total	**100 μL**	
Store at 4°C until use and protect from light.		

Antibodies cocktail n°2 – Intracellular markers post-restimulation – 1× concentration		
Reagent (stock concentration)	Amount	Working dilution (in antibodies cocktail)
RORγt PE-eFluor 610 (0.2 mg/mL)	0.5 μL	1:200
Foxp3 PE-Cyanine5.5 (0.2 mg/mL)	0.5 μL	1:200
IL-10 Alexa Fluor 488 (0.5 mg/mL)	1.0 μL	1:100
IL-17A APC/Cyanine7 (0.2 mg/mL)	1.0 μL	1:100
ChromPure Rat IgG* (11.6 mg/mL)	2.0 μL	1:50
Permeabilization buffer** (1×)	Up to 100 μL	
Total	**100 μL**	
Store at 4°C until use and protect from light.		
*See CRITICAL text on step 13.		
** From eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set.		

STEP-BY-STEP METHOD DETAILS

Retrovirus production – Days 1–4

Timing: 4 days

This section outlines the steps for producing retroviral constructs encoding expression cassettes for Thy1.1 or NGFR in Plat-E cells.

1. Seeding Plat-E cell working culture – Day 1.

⚠ CRITICAL: Ensure Plat-E culture medium does NOT contain penicillin-streptomycin before transfection.

   a. Once the pre-established Plat-E cell stock culture (pre-passaged 1–2×) has been grown to ~80% confluency, gently remove culture media and wash cells with 5 mL RT sterile PBS 1×.
   b. Gently apply 1–2 mL trypsin-EDTA 0.05% solution and spread across the plate or flask to ensure even coverage.
   c. Incubate plate or flask in a 5% CO_2 incubator at 37°C for 3–5 min to trypsinize cells.
   d. Add 5 mL RT Plat-E medium to inhibit further digestion.
   e. Transfer cell suspension into a 15 mL conical tube and centrifuge at 500 × *g* for 5 min, RT.
   f. Remove supernatant and resuspend cells with 5 mL RT Plat-E medium.
   g. Determine the viable cell concentration of the resuspension using a hemocytometer or automated cell counter (e.g., Vi-cell cell counter).
   h. In a 6-well tissue culture plate, seed 0.5 × 10^6 viable Plat-E cells in 1.25 mL RT Plat-E medium into two wells:
      i. Well #1: "Thy1.1 retrovirus"—For the production of Thy1.1-encoding retroviral constructs.
      ii. Well #2: "NGFR retrovirus"—For the production of NGFR-encoding retroviral constructs.
   i. Incubate at 37°C in a 5% CO_2 incubator overnight.

2. Plat-E cell transfection with plasmid DNA – Day 2.

Note: Be careful to avoid generating bubbles when mixing by pipette.

   a. Make Mixture "A" by adding 250 μL of RT Opti-MEM™ reduced serum medium and 15 μL of RT Mirus transfection reagent into a 1.5 mL tube. Mix well by pipetting. Incubate at RT for 5 min.
   b. Make a separate Mixture "B" for Thy1.1 or NGFR transfection each by mixing 125 μL of Opti-MEM™ with 2.5 μg of either Thy1.1- or NGFR-plasmid DNA into a separate 1.5 mL tube. Mix well by pipetting.
   c. Carefully pipette 125 μL of Mixture "A" into each Mixture "B" tube and mix well by pipetting. Now there should be two transfection mixtures: one for Thy1.1 transfection and one for NGFR transfection, each containing ~250 μL.
   d. Incubate the transfection mixtures at RT for 18 min.
   e. Homogenize each transfection mixture by carefully pipetting up and down 3–5 times.
   f. Use a P200 pipette to evenly distribute each transfection mixture dropwise onto their corresponding well:
      i. Distribute the Thy1.1 transfection mixture onto Well #1: "Thy1.1 retrovirus".
      ii. Distribute the NGFR transfection mixture onto Well #2: "NGFR retrovirus".
   g. Gently swirl the plate to mix and incubate for 16–20 h at 37°C in a 5% CO_2 incubator.

3. Culture medium change – Day 3.
   a. Gently remove the transfection mixture-containing medium from each well.
   b. Add 2 mL RT IMDM complete medium to each well.
   c. Incubate the transfected cells for a further 24 h at 37°C in a 5% CO_2 incubator.

4. Retrovirus harvest – Day 4.
   a. Aspirate the 2 mL of retrovirus-containing medium from each well into a 3 mL syringe and pass through a 0.45 μM syringe filter (we use the Thermofisher™ Millex-HV 0.45 μM Syringe Filter Unit) to remove large debris from the packaging cell line.
   b. Aliquot 0.5 mL of the retrovirus-containing medium into a clean 1.5 mL tube on ice.
   c. Each 0.5 mL aliquot of retrovirus-containing medium is now ready to be used immediately for a single naïve $CD4^+$ T-cell transduction (step 9).

Note: Virus-containing tubes can be stored frozen at −80°C for later use (up to 6 months).

Note: For best transduction efficiency, opt to use freshly harvested (never frozen) retrovirus-containing medium for naïve $CD4^+$ T-cell transduction. This protocol assumes this recommendation is followed.

Naïve $CD4^+$ T cell isolation and activation – Day 3

Timing: 5–6 h

In the following section, naïve $CD4^+$ T cells are isolated from murine spleens. Cells are seeded onto a 24-well plate pre-coated with anti-hamster IgG, which will help spatially cluster the hamster anti-mouse CD3ϵ and anti-mouse CD28 antibodies to activate naïve T cells, as described in (Ciofani et al., 2012; Ma et al., 2022). This system requires fewer antibodies for more effective T cell activation than direct coating with anti-CD3ϵ and anti-CD28.

5. Pre-coat 24-well plate.
   a. Dilute anti-hamster IgG stock solution (1 mg/mL) at 1:20 with sterile PBS (1×).
   b. Add 240 μL diluted anti-hamster IgG (0.05 mg/mL) to four wells of a 24-well plate.
   c. Incubate the plate for 2 h at 37°C in a 5% CO_2 incubator.

   Alternatives: The 24-well plate can be pre-coated in advance, sealed with parafilm (to prevent evaporation), and incubated at 4°C at least overnight for up to 1 week as needed.

6. Naïve $CD4^+$ T cell isolation and activation.
   a. Euthanize mice according to applicable IACUC guidelines. Here, we use $ROR\gamma t^{WT}$ and $ROR\gamma t^{S182A}$ cohoused littermates (one of each) of the same gender.
   b. Take the spleen from each mouse and place it onto a 40 μM cell strainer on top of a 6-well plate with 5 mL IMDM complete medium in each well.
   c. Use a plunger from a sterile 3 mL syringe to grind each spleen into a single cell suspension.
   d. Transfer the single-cell suspension to a 15 mL falcon tube and centrifuge at 650 × *g* for 5 min at 4°C to pellet splenic cells.
   e. Carefully remove the supernatant and add 1 mL ACK lysis buffer to each cell pellet to eliminate red blood cells. Mix well by pipetting and incubate at RT for 1 min.

   Note: Avoid incubating cells in ACK lysis buffer for longer than 1 min to prevent death of lymphocytes.

   f. Add 9 mL MACS buffer to halt further cell lysis, and centrifuge at 650 × *g* for 5 min at 4°C.
   g. Decant supernatant. The cell pellet should appear white as an indication of successful red blood cell lysis and removal.
   h. Proceed to purify naive $CD4^+$ T cells from the cell pellet using the Naive $CD4^+$ T Cell Isolation Kit according to the manufacturer's instructions.

   Note: Each spleen should yield ∼2–10 × 10^6 naïve $CD4^+$ T cells.

   i. Wash the four precoated wells of the 24-well plate (from step 5) with 500 μL sterile PBS (1×) or IMDM medium.
   j. In two wells each, seed 0.2–0.3 × 10^6 $ROR\gamma t^{WT}$ or $ROR\gamma t^{S182A}$ naïve $CD4^+$ T cells in 500 μL IMDM complete medium together with anti-CD3ϵ and anti-CD28 at a final concentration of 0.25 μg/mL and 1 μg/mL, respectively.
   k. Incubate at 37°C in a 5% CO_2 incubator for 24 h.

Note: Compared to RPMI, IMDM showed better performance in inducing PMA/Ionomycin-mediated activation of $CD4^+$ T cells (Zimmermann et al., 2015). This media has been adopted by multiple recent T cell studies (Amir et al., 2018; Ciofani et al., 2012; Ma et al., 2022; Veldhoen et al., 2009).

Note: We omit the addition of IL-2 to the IMDM T-cell activation mixture as it was previously found to negatively impact the generation of Th17 cells in culture assays (Laurence et al., 2007; Liao et al., 2011).

Retroviral transduction – Day 4

Timing: 2 h

In this section, Thy1.1 or NGFR retroviral constructs are used to transduce, and label activated $CD4^+$ T cells with Thy1.1 or NGFR surface markers.

⚠ CRITICAL: Transduction of activated $CD4^+$ T cells occurs optimally during the mitotic phase of the cell cycle, which corresponds to 24–48 h post-anti-CD3ϵ and anti-CD28 stimulation.

7. Add 4 μg polybrene per aliquot to two 0.5 mL aliquots of Thy1.1 or NGFR retrovirus medium each (prepared in step 4). Mix well by pipetting.

Note: If the retrovirus medium was previously frozen, it must be thawed on ice for 1–2 h before the addition of polybrene.

8. Gently remove the medium from all wells seeded with $ROR\gamma t^{WT}$ or $ROR\gamma t^{S182A}$ $CD4^+$ T cells of the 24-well plate prepared in step 6.
9. Add a 0.5 mL aliquot of Thy1.1 retrovirus medium to one well of $ROR\gamma t^{WT}$ $CD4^+$ T cells and one well of $ROR\gamma t^{S182A}$ $CD4^+$ T cells each. Do the same for the NGFR retrovirus medium.
10. Spin inoculates by centrifuging the plate at 900 × *g* for 90 min at 32°C with an acceleration rate of 3 and a deceleration rate of 1.

⚠ CRITICAL: Centrifugation at 32°C is crucial for transduction as previously described (Eremenko et al., 2021; Pampusch et al., 2020).

⚠ CRITICAL: The retrovirus-containing plate should be sealed with plastic wrap before centrifugation to avoid the release of retroviral particles into the centrifuge.

11. Gently remove the plastic wrap (if used) from the plate and incubate overnight at 37°C in a 5% CO_2 incubator.

Ex vivo T cell co-culture and polarization – Days 5–8

Timing: 4 days

Transduced Thy1.1- or NGFR-expressing $CD4^+$ T cells are co-cultured and polarized *ex vivo* as previously described (Ma et al., 2022).

12. Setup $CD4^+$ T cell co-culture – Day 5.
    a. Add 40 μL/well of diluted anti-hamster IgG (same as described in step 5) into two wells of a 96-well plate.

b. Incubate the plate for 2 h at 37°C in a 5% CO_2 incubator.
c. Prepare the T-cell polarization medium as outlined under "materials and equipment".

Note: The cytokine combination comprising the T-cell polarization medium was chosen to closely mimic the T lymphocyte populations observed in the intestinal lamina propria—namely a mixture of Th17 (Th17, RORγt$^+$Foxp3$^-$), RORγt$^+$ Treg (RORγt$^+$Foxp3$^+$), and conventional Treg (cTreg, RORγt$^-$Foxp3$^+$) lineages (Figure 4). IL-6 and TGFβ alone—without the addition of anti-IFNγ and anti-IL-4—are sufficient to effectively polarize naïve CD4$^+$ T cells toward the Th17 lineage (Bedoya et al., 2013). To induce concomitant polarization towards RORγt$^+$ Treg and RORγt$^-$ cTreg lineages, we utilize TGFβ at 5 ng/mL, a dose known for polarizing naïve CD4$^+$ T cells toward the Treg lineages in culture (Shevach et al., 2008). IL-1β (20 ng/mL) is used to mimic an inflammatory tissue environment, such as that of the colon post Dextran Sodium Sulfated (DSS) induced epithelial injury (Ma et al., 2022).

Alternatives: Cytokine combinations/concentrations can be adjusted as desired to obtain polarization towards specific T-helper subsets.

d. Gently remove the retrovirus-containing medium from each well of the T-cell transduction plate from step 11.
e. Use 0.5 mL T-cell polarization medium to resuspend transduced T-cells from each well by gentle pipetting and transfer to a fresh 1.5 mL tube.
f. Count cells as described in step 1.g.
g. After the 2-h incubation of the 96-well plate, remove the anti-hamster IgG solution and wash both pre-coated wells with 100 μL PBS or IMDM medium.
h. Seed Thy1.1- and NGFR-expressing cells at a 1:1 ratio into both wells for a combined cell density of 0.08–0.12 × 10^6 cells per well (as illustrated in Figure 2):
   i. Group #1: 100 μL NGFR-transduced RORγt^{WT} T-cells + 100 μL Thy1.1-transduced RORγt^{S182A} T-cells.
   ii. Group #2: 100 μL Thy1.1 transduced RORγt^{WT} T-cells + 100 μL NGFR-transduced RORγt^{S182A} T-cells.
i. Incubate the plate for 48–72 h at 37°C in a 5% CO2 incubator.

Note: To avoid overgrowth if cells need to be cultured for longer than 72 h before restimulation, cells should be split into new pre-coated wells at a 1:2–1:4 ratio after 72 h.

13. T-cell restimulation and FACS staining – Day 7 or 8.
   a. Add restimulation cocktail (final concentrations: 5 ng/mL PMA, 500 ng/mL ionomycin, and 0.625 μL/mL GolgiStop) to each well. Mix well by pipette.
   b. Incubate for an additional 4–5 h at 37°C in a 5% CO_2 incubator.

Note: For the following steps as part of the experiment illustrated in Figures 3 and 4, we followed the manufacturer's instructions suggested for the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set.

c. Centrifuge the cells at 600 × *g* for 5 min at 4°C and decant supernatant.
d. Wash the cells with 150 μL PBS 1×. Centrifuge and decant supernatant as before.
e. Add 40 μL of antibodies cocktail n°1 to each well to stain for surface markers. Mix well by pipetting.
f. Incubate the cells for 30 min at 4°C in the dark.
g. Wash the cells with 150 μL MACS buffer. Centrifuge and decant supernatant as before.
h. Fix and permeabilize the surface-stained cells with 40 μL fixation/permeabilization solution (from eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set) for 30 min or overnight at 4°C in the dark.

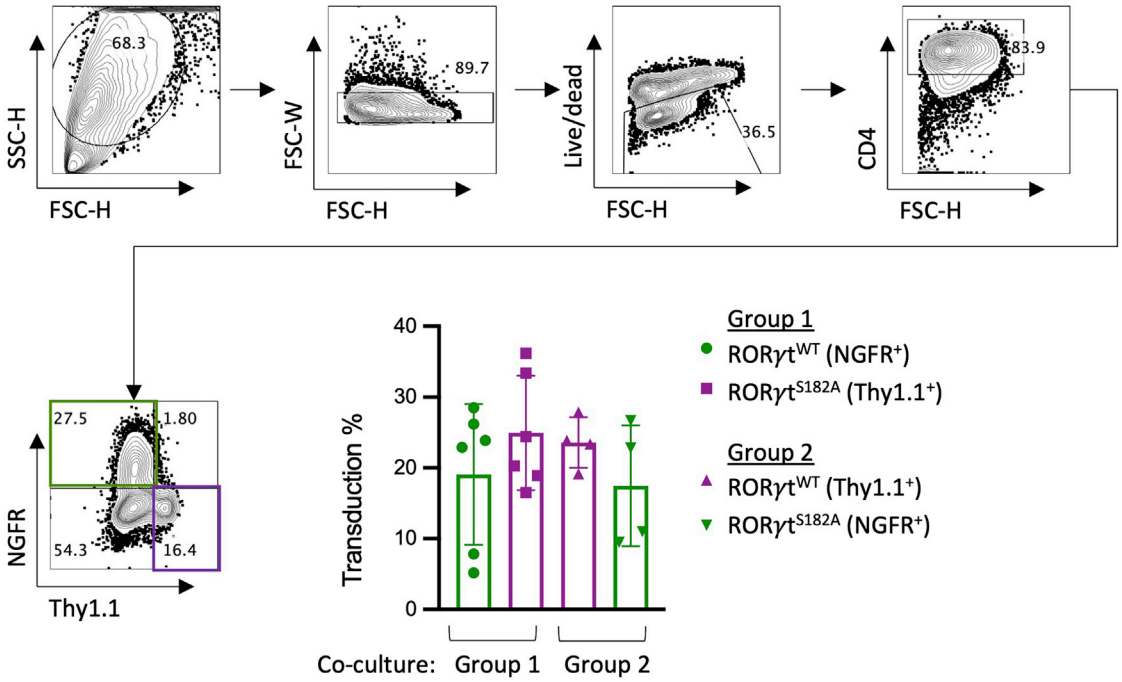

Figure 3. Expected transduction efficiency in cultured murine CD4$^+$ T cells

Top: Flow cytometry gating strategy for evaluating transduction efficiency in live CD4$^+$ T cells. Bottom: Proportions of live NGFR$^+$ or Thy1.1$^+$ CD4$^+$ T cells 72 h post-polarization. Data are represented as mean ± SD.

i. Wash cells with 150 µL Permeabilization buffer 1× (from eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set) per well.
j. Centrifuge the cells at 1000 × *g* for 5 min at 4°C and decant supernatant.
k. Repeat step 13. i-j once.

⚠ CRITICAL: After cell fixation/permeabilization, the centrifugation speed should be increased to 1000 × *g* (from step 13. j onward) to avoid cell loss after decanting of supernatant.

l. Add 40 µL of antibodies cocktail n°2 to each well to stain for intracellular transcription factors/cytokines. Mix well by pipetting.

⚠ CRITICAL: To reduce the potential background of RORγt staining, ChromPure Rat IgG (1:50) should be included in antibodies cocktail n°2.

m. Incubate the cells for 60 min at 4°C in the dark.
n. Wash cells with 150 µL Permeabilization buffer 1× per well.
o. Centrifuge the cells at 1000 × *g* for 5 min at 4°C and decant supernatant.
p. Repeat step 13. n-o once.
q. Resuspend cells in 100–200 µL of MACS Buffer or PBS 1× per well.
r. Measure the fluorescence intensity of each labeled cell using a flow cytometer (we used the Bio-Rad ZE5 Cell Analyzer).

EXPECTED OUTCOMES

The retroviral transduction efficiency of naïve CD4$^+$ T cells ranges from 15%–30% (Figure 3). 72 h incubation of transduced CD4$^+$ T cells in our T-cell polarization medium yields ~50% Th17 cells

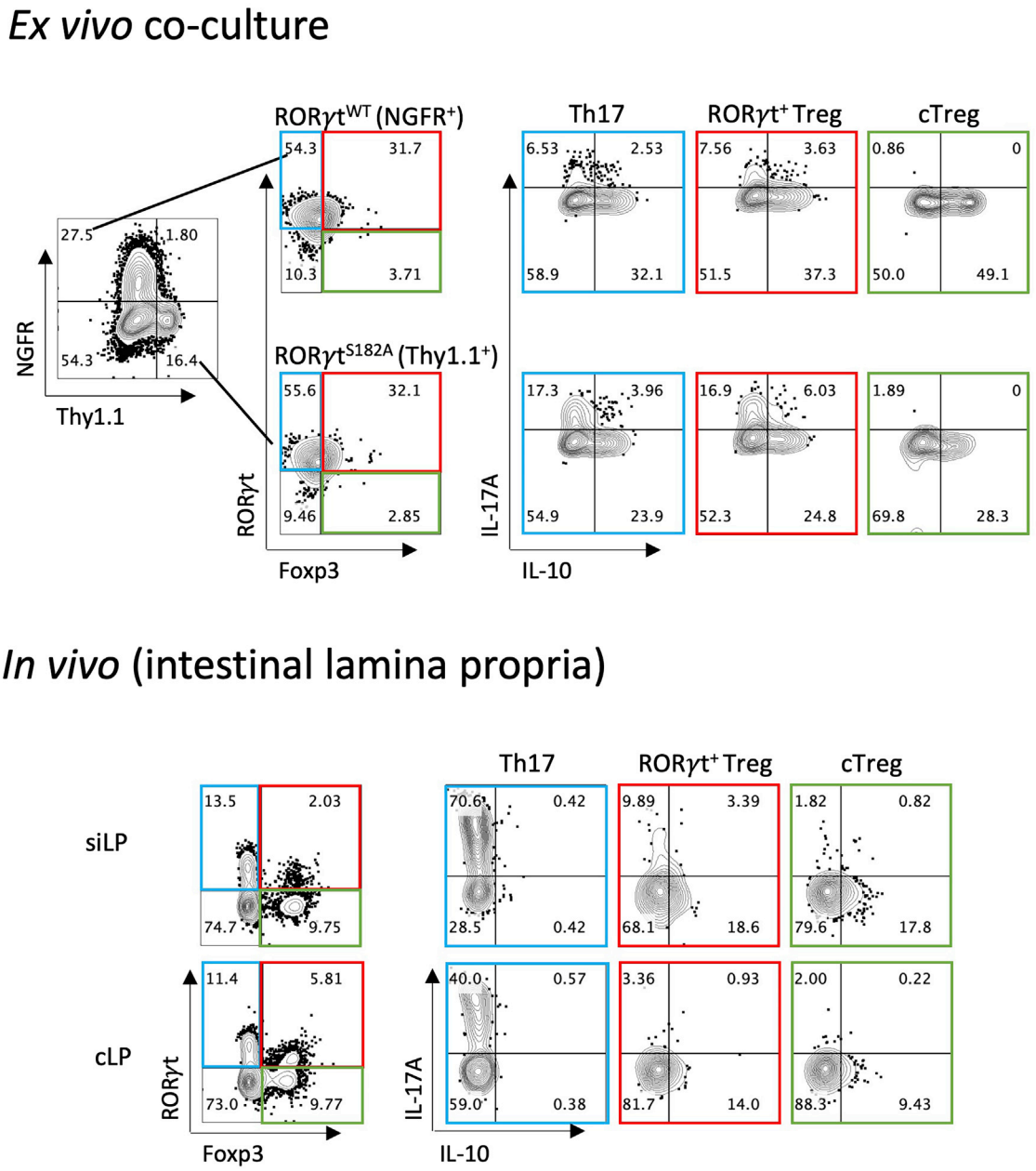

Figure 4. Comparison of *ex vivo* co-culture and *in vivo* intestinal lamina propria T lymphocyte populations

Top: Representative flow cytometry plots of transduced RORγt^{WT} (NGFR$^+$) and RORγt^{S182A} (Thy1.1$^+$) CD4$^+$ T cells co-cultured and polarized toward multiple T cell subsets, including Th17 (RORγt$^+$Foxp3$^-$, blue), RORγt$^+$ Treg (RORγt$^+$Foxp3$^+$, red) and cTreg (RORγt$^-$Foxp3$^+$, green). Bottom: Representative flow cytometry plots from the small intestinal and colonic lamina propria (siLP and cLP), respectively. Lamina propria T lymphocyte populations were isolated as previously described by (Lefrancois and Lycke, 2001; Valle-Noguera et al., 2020). Note that IL-10 staining on *ex vivo* (top) and *in vivo* (bottom) samples was performed with clone JES5-16E3 (Alexa Fluor 488) and JES5-16E3 (Alexa Fluor 700), respectively.

(RORγt$^+$Foxp3$^-$), ~30% RORγt$^+$ Tregs (RORγt$^+$Foxp3$^+$), and ~5% cTregs (RORγt$^-$Foxp3$^+$) (Figure 4, top panel). These *ex vivo* differentiated T-cell subsets harbor IL-17A and IL-10 production capabilities like those obtained *in vivo* (Figure 4, bottom panel).

In our published study, we found that CD4$^+$ T subsets from RORγt^{S182A} mice displayed an increase in IL-17A production and a decrease in IL-10 production compared to the cells from RORγt^{WT} mice. In

the co-culture assay (Figure 4), RORγt^{S182A} T cell subsets co-cultured in the presence of RORγt^{WT} cells also maintained an increased IL-17A production capacity and a reduced IL-10 production capacity. This suggests that RORγt^{S182} regulates CD4^{+} T cell effector functions in a cell-intrinsic manner (Ma et al., 2022). If the candidate molecules under investigation exert paracrine roles in driving T cell differentiation and/or function, we would instead observe co-cultured control and mutant cells displaying similar differentiation and cytokine patterns.

QUANTIFICATION AND STATISTICAL ANALYSIS

Un-transduced T cells can be used as a flow gating control for identifying the retrovirus transduced T cell population. Isotype controls can be used to decipher a gating strategy for identifying transcription factor and cytokine positive populations on the flow cytometer. Summarized graphs should be presented as means ± standard deviation. Significant differences can be evaluated using the student's t-test, paired t-test, or multiple t-tests. A two-tailed p-value of <0.05 is considered statistically significant in all experiments.

LIMITATIONS

Retrovirus is not suitable for the transduction of non-proliferating cells. Caution must be exercised for retrovirus transduction of mutant T-cells that have diminished or loss of proliferative capacity (Cepko and Pear, 2001; Eremenko et al., 2021). Retrovirus transduction can substantially negatively impact T-cell viability. Providing transduced CD4^{+} T cells with recombinant IL-2 and IL-7 may increase their viability and proliferative capacity as previously described (Eremenko et al., 2021), but may negatively impact polarization towards the desired T-helper subsets. In addition, retrovirus transduction efficiency is relatively low (combined NGFR and Thy1.1 transduction efficiencies are ~40%–60%) in our system. Furthermore, the T-cell polarization conditions described above typically yield only ~10%–30% RORγt^{+} Tregs and <10% cTreg cells. Polarization conditions need to be tailored for individual studies before performing large-scale experiments.

TROUBLESHOOTING

Problem 1

Plat-E cells fail to grow after transfection with plasmid DNA (steps 2 and 3).

Potential solution

Transfecting excessive amounts of plasmid DNA can negatively impact Plat-E cell growth. Reduce the amount of plasmid DNA used for transfection.

Problem 2

Low Plat-E transfection efficiency (steps 2 and 3).

Potential solution

- Do not transfect freshly thawed Plat-E cells. Cells require sufficient time to fully recover from the freeze-thaw process.
- Do not add penicillin-streptomycin into the Plat-E culture medium.
- After transfection, avoid physically disturbing the plate as this could detach Plat-E cells from the bottom. Pre-coating plates with poly-D-lysine can enhance the adherence of Plat-E cells.
- Repeat transfections can be carried out the subsequent day to further improve transfection efficiency.

Problem 3

Low transduction efficiency (steps 7–11).

Potential solution

- Adjust the transduction window of T cells, e.g., delaying the time of transduction to 48 h post-activation as described for CD8 cells (Kurachi et al., 2017).
- Increase transduction virus titers.
- Reduce the naïve T-cell seeding density in the 24-well plate.

Problem 4

Low $CD4^+$ T cell viability post-transduction (steps 9–11)

Potential solution

- Replace retrovirus-containing medium with 500 μL fresh IMDM complete medium 4–6 h post-transduction to provide optimal nutrients to the T cells.
- Avoid plating excessive numbers of T-cells, which can quickly deplete nutrients. Follow the recommended seeding density outlined in the Methods section.
- Use an optimal polybrene concentration of 6–16 μg/mL.
- Confirm centrifugation settings. Centrifugation time can be increased to 2–3 h to ensure retroviral enrichment at the bottom of the well where the cultured cells reside.

Problem 5

Low $CD4^+$ T cell activation/polarization (steps 12–13).

Potential solution

- Confirm appropriate cytokine and antibody concentrations were used.
- Avoid freeze-thaw cycles on cytokines and antibodies. Typically, stock concentrations of cytokines and antibodies are 20–100 mg/mL and 1–10 mg/mL, respectively. These should be stored in 5–20 μL aliquots in the −80°C freezer according to manufacturer recommendations.
- Ensure culture media components have not expired.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wendy Jia Men Huang (wendyjmhuang@ucsd.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not report datasets and code.

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AUTHOR CONTRIBUTIONS

S.M. designed, performed the experiments, and wrote the manuscript. J.E.H. and W.J.M.H. edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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